# ISOLATION AND SCREENING OF LACTIC ACID BACTERIA FROM FERMENTED MILK PRODUCTS FOR BACTERIOCIN PRODUCTION

Sani Sambo Datsugwai Mohammed[*1], Udeme Josiah Joshua Ijah[2]
[*1] Department of Microbiology, Faculty of Natural Sciences, Ibrahim Badamasi Babangida University,P.M.B.11,Lapai, Niger State,Nigeria
[2] Department of Microbiology, Federal University of Technology,P.M.B.65, Minna, Niger State,Nigeria
* E-mail: : mosada78@yahoo.com

***Abstract***
*Isolation of lactic acid bacteria (LAB) from yoghurt, wara (cheese) and nono (fermented milk) was carried out. LAB were cultivated on lactic acid medium and were characterized based on colony morphology, cell morphology and biochemical tests. Out of fifteen samples analysed, thirteen (86.6%) harboured LAB. Nono had the highest LAB counts (9.8 x $10^6$ cfu/ml) while yoghurt had the lowest LAB counts (3.1x$10^6$ cfu/ml). The lactic acid bacterial isolates were identified as Lactobacillus bulgaricus (31.6%), Lactobacillus lactis (15.8%), Lactobacillus acidophilus (10.5%), Streptococcus thermophilus (15.8%), Strectococcus cremoris (10.5%), Lactococcus lactis (15.8%), Pediococcus halophilus (5.3%) and Pediococcus cerevisiae (5.3.%). The presence of these organisms in fermented milk products and other food products will enhance preservation ( shelf life extension) of the products. The LAB were screened for potential to produce bacteriocins in De Man Rogosa Sharpe (MRS) broth. Of the thirty four LAB screened for bacteriocin production, nineteen (55.9%) were potential bacteriocin producers and they included strains of Lactobacillus bulgaricus, Lactobacillus lactis, Lactobacillus acidophilus, Lactococcus lactis, Streptococcus cremoris, Pediococcus halophilus and Pedioccus cerevisiae. Lactobacillus bulgaricus had the highest bacteriocin activity of 6000 Au/ml against all the indicator microorganisms used while Pediococcus cerevisiae had the least bacteriocin activity of 4800 Au/ml against salmonella sp and Bacillus sp.The presence of LAB in foods can cause shelf life elongation and safety of the food products.*

**Keywords:** lactic acid bacteria, bacteriocins, fermented milk, De Man Rogosa Sharpe broth, lactic acid medium

Submitted: 03.02.2013 Reviewed: 09.04.2013 Accepted: 13.05.2013

## 1. INTRODUCTION

Lactic acid bacteria (LAB) are group of gram-positive, non spore forming, non–respiring, cocci or rods, which produce lactic acid as the major end product of the fermentation of carbohydrate (Axelson, 1998). LAB are used as natural or selected starters in food fermentation in which they perform acidification due to production of lactic acid flavor. Bacteria from the genera *Lactobacillus, Lactococcus, Leuconostoc, Pediococcus* and *Streptococcus* are the main species of LAB involved. Although several more have been identified but they play a minor role in lactic fermentations (Axelson, 1998). LAB are often inhibitory to other microorganisms and this is the basis of their ability to affect the keeping quality and safety of many food products. The principal factors, which contribute to this inhibition, are low pH, organic acids bacteriocins, hydrogen peroxide, ethanol, nutrient depletion and low redox potential. By far the most important are the production of lactic acid and acetic acid and the consequent decrease in pH (Adams and Nicolaides, 1997). Olukoya (1993) in his study on 'nono' equally isolated *Lactobacillus acidophilus, Streptococcus lactis* and *Staphylococcus* sp. *Lactobacillus, Streptococcus and Staphylococcus* sp were isolated from milk products (Casla *et al*; 1996). Oyeleke *et al.* (2006) in their study on 'nono', 'wara', 'mai – shanu', 'fufu' and 'kamu' isolated *Lactobacillus bulgaricus, L. lactis, L. acidophilus, Streptococcus thermophilus* and *S. cremoris.* Olatunji *et al* .(2006) reported that wara has been found to habour bacteria including LAB such as *Lactobacillus, Streptococcus* sp as well as yeast and molds.
Bacteriocins are naturally occurring antibiotic peptides produced by Gram-positive bacteria, they are usually small such as 24 amino acids

(Chatterjee *et al.*, 2005). Many bacteriocin are active against food-born pathogens especially against *Listeria monocytogenes* (Vignolo *et al*, 1996). Several types of bacteriocins from food – associated lactic acid bacteria have been identified and characterized, of which the important ones are nisin, diploccocins, acidophilins, bulgarican, helveticins, lacticins and plantaricins (Nettles and Bare-foot, 1993).
Vignolo *et al*. (1995) reported that maximal bacteriocin production could be obtained by supplementing a culture medium with growth limiting factors such as sugars vitamins and nitrogen sources or by regulating pH of the growth medium or by choosing the best adapted culture medium.
At present, nisin produced by *Lactococcus lactis* is the only bacteriocin commercially available and marketed (Balasubramayam and varadaraj, 1998). It has been reported that nisin is more effective against Gram-positive bacteria, particularly the spore formers (Delves- Broughton, 1990). Other bacteriocins of *Lactobacillus* has been reported to be effective against closely related species of mesophilic *Lactobacillus* and therefore considered as potential natural food preservatives (Daeschel, 1993., De Vugst and Vandamme, 1994).
The aim of this study is to isolate and screen lactic acid bacteria in some fermented milk products for potentials to produce bacteriocin. Their presence in foods will enhance shelf life elongation .

## 2. MATERIALS AND METHODS

### Collection of samples

Five (5) samples each of yoghurt, wara and nono were collected in sterile sample bottles from Bosso market, Minna, Niger State, Nigeria and transferred to the laboratory for isolation of Lactic acid bacteria (LAB) and other analysis.

### Isolation and Identification of Lactic Acid Bacteria

Serially diluted samples of the fermented milk products (yoghurt, wara and nono) were inoculated on to lactic acid agar medium, LAM (agar-agar 15g, tryptone 20g, yeast extract 5g, gelatin 2.52g, glucose, 5g, lactose 5g and sucrose 0.52g) aseptically and separately incubated at $37^0$C for 24-48 hours. Colonies that appeared on the agar plates were counted using colony counter (model 6399, Stuart scientific Co. Ltd, Great Britain) and the results were recorded as colony forming units per milliliter (cfu/ml) or gramme (cfu/g) of sample. Pure isolates were characterized based on colony morphology, cell morphology and biochemical tests (Fawole and Oso; 1998; Oyeleke and Manga, 2008). The isolates were identified using the scheme of cheese brough (2003).

### Screening of lactic acid bacteria for bacteriocin production

Lactic and bacteria were propagated in 1000ml of De Man Rogosa Sharpe (MRS) broth (pH 5.8) at $30^o$C for 48 hours. For extraction of bacteriocins, a cell free solution of bacteriocin was obtained by centrifugation at 10,000 rpm for 20 minutes. The culture was adjusted to pH 7.0 using 1M NaOH to exclude the antimicrobial effects of organic acid, followed by filtration of the supernatant through 0.2µm pore size cellulose acetate filter (Schillinger and Lucke, 1989) to obtain crude bacteriocin for each sample. Inhibition activity from hydrogen peroxide ($H_2O_2$) was eliminated by the addition of 5mg/ml catalase (Daba *et al*., 1991). The cell free broth culture was tested (screened for pH growth of producer organisms and bacteriocin activity against the indicator (test) micro organisms (Brinkten *et al*, 1994., Graciela *et al.,* 1995).
The growth of producer organism was determined when the broth culture of the bacteriocin produced was subjected to colometric analysis, at ware length of 580nm. (Brinkten *et al*., 1994). To determine the bacteriocin activity, well assay procedure of Schillinger and Lucke (1989) and Takuhiro *et al*. (1991) was used. Aliquots of 50µl from each bacteriocin produced was placed in agar wells in Petri dishes seeded with the bio assay strain (indicator microorganism): *Staphyloccus*

sp, *Salmonella* sp, *Shigella* sp, *Bacillus* sp, and *Pseudomonas* sp) and incubated overnight at 37°C. The millimeter of the zone of inhibition (mm) was measured (Rammelsberg and Radlar, 1990). The antimicrobial activity of the bacteriocins produced was defined as the reciprocal of the highest dilution showing inhibition of microorganisms multiplied by 100 and it is expressed as activity unit per milliliter (Au/ml) (Graciela *et al*., 1995).

## 3. RESULTS

### Occurrence of lactic and bacteria in fermented milk products

Thirteen (86.6%) out of fifteen fermented milk products (yoghourt, nono and wara) analysed contained lactic acid bacteria (LAB). The result (Table 1) showed that nono had the highest counts ($3.2 \times 10^6$-$9.8 \times 10^6$ (cfu/ml) followed by wara ($1.7 \times 10^6$-$3.3 \times 10^6$cfu/g) while yoghurt had the least count ($1.1 \times 10^6$-$3.1 \times 10^6$cfu/ml). The LAB were identified as species of *Lactobacillus, Streptococcus, Lactococcus* and *Pediococcus* (Table 2). *Pediococcus* and *Lactococcus* were not detected in yoghurt. Similarly *Lactococcus* was not detected in wara. Nono had all four genera listed above. *Lactobacillus bulgaricus* were more frequently isolated and constituted 31.6% of the total isolates obtained, followed by *Lactobacillus lactis* and *Streptococcus thermophilus* with 15.8% each. *Lactobacillus acidophilus* and *Streptococcus cremoris* had 10.5% each while *Lactococcus lactis, Pediococcus halophilus* and *Pediococcus cerevisiae* had 5.3% frequently of occurrence each (fig. 1).

**Table1. Counts of lactic acid bacteria in fermented milk products**

| Fermented milk products | Range Of counts of LAB |
|---|---|
| Yoghurt | $1.1 \times 10^6$ - $3.1 \times 10^6$cfu/ml |
| Wara | $1.7 \times 10^6$-$3.3 \times 10^6$cfu/g |
| Nono | $3.2 \times 10^6$-$9.8 \times 10^6$cfu/ml |

LAB: Lactic acid bacteria.

**Table2. Presence of lactic acid bacteria in fermented Milk Products**

| Lactic acid bacteria | Fermented milk products | | |
|---|---|---|---|
| | Yoghurt | Wara | Nono |
| *Lactobacillus bulgaricus* | + | - | + |
| *Streptococcus thermophilus* | + | - | + |
| *Pediococcus halophilus* | - | + | - |
| *Streptococcus cremoris* | - | + | - |
| *Lactobacillus lactis* | - | + | - |
| *Lactococcus lactis* | - | - | + |
| *Lactobacillus acidophilus* | - | - | + |
| *Pediococcus Cerevisiae* | - | - | + |

+: Present
-: Absent

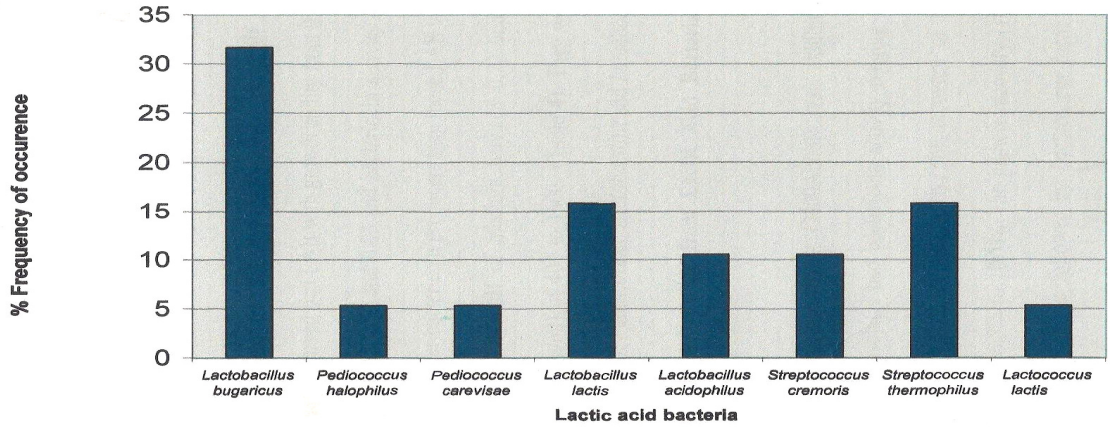


**Fig 1. Frequency of occurrence of lactic acid bacteria in fermented milk products**

**Table 3. Growth, pH and bacteriocin producing ability of lactic acid bacteria**

| Coded Isolates | Growth (580nm) | pH of (bacteriocin) Medium | Bacteriocin Activity (Au/ml) |
|---|---|---|---|
| *S. cremoris* W1 | 0.80 | 3.90 | 5300* |
| *L. helveticus* W2 | 0.32 | 1.80 | 1600 |
| *S. lactis* W3 | 0.20 | 1.10 | 1000 |
| *Leuconostoc* sp W4 | 0.25 | 1.21 | 1200 |
| *S. faecalis* W5 | 0.10 | 1.00 | 800 |
| *S. faecalis* W6 | 0.11 | 1.01 | 800 |
| *P. domnosus* W7 | 0.09 | 0.70 | 400 |
| *S. lactis* W8 | 0.20 | 1.00 | 1000 |
| *P. domnosus* W9 | 0.72 | 3.80 | 5000* |
| *P. halophilus* W10 | 0.09 | 0.72 | 400 |
| *S. faecalis* W11 | 0.10 | 1.00 | 800 |
| *S. cremoris* W12 | 0.75 | 3.71 | 5300* |
| *Leuconostoc sp.* W13 | 0.25 | 1.20 | 1200 |
| *S. faecalis* W14 | 0.10 | 1.00 | 800 |
| *L. lactis* W15 | 0.85 | 4.03 | 6000* |
| *S. lactis* W16 | 0.21 | 1.00 | 1000 |
| *L. acidophilus* N17 | 0.90 | 3.70 | 5600* |
| *L. bulgaricus* N18 | 0.89 | 3.90 | 5800* |
| *P. domnosus* N19 | 0.09 | 0.70 | 400 |
| *L. acidophilus* N20 | 0.90 | 3.71 | 5600* |
| *P. cerevisae* N21 | 0.78 | 3.81 | 4800* |
| *Lactoccus lactis* N22 | 0.90 | 3.70 | 5600* |
| *Lactococcus lactis* N23 | 0. 89 | 3.71 | 5600* |
| *Lactococcus lactis* N24 | 0.90 | 3.72 | 5600* |
| *S. thermophilus* N25 | 0.82 | 4.01 | 5400* |
| *L. bulgaricus* Y26 | 0.90 | 3.91 | 5800* |
| *S. thermophilus* Y27 | 0.82 | 4.00 | 5400* |
| *S. faecalis* Y28 | 0.11 | 1.01 | 800 |
| *S. thermophilus* Y29 | 0.83 | 4.02 | 5400* |
| *S. lactis* Y30 | 0.20 | 1.00 | 1000 |
| *L. bulgaricus* Y31 | 0.88 | 3.91 | 5800* |
| *L. bulgaricus* Y32 | 0.90 | 3.92 | 5800* |
| *L. bulgaricus* Y33 | 0.91 | 3.90 | 5800* |
| *L. bulgaricus* Y34 | 0.90 | 3.91 | 5800* |

**W**: wara, **N**: nono, **Y**: yoghurt, Au/ml: Activity per milliliter, nm: nanometer
*: potential bacteriocins produced.

*Listeria monocytogenes* and *Enterococcus faecalis.*

Earlier El-shafei *et al.* (2000) screened one hundred lactic acid bacterial strains isolated from traditional fermented foods (yoghurt, milk, sour cream, sour dough, cheese) for bacteriocin production and found twenty six strains producing a nisin-like bacteriocins. Most of these isolates gave only a narrow inhibitory spectrum. Although one showed a broad inhibitory activity against the indicator strains selected.

Bacteriocins produced by *L. bulgaricus* Y34, *L. lactis* N22 and *S. thermophilus* Y27 had some interesting characteristics. The most stricking is that this bacteriocins were limited by extreme broad antimicrobial spectrum which is similar to the report for some bacteriocin of LAB with narrow spectrum for example Lactococcin A (Halo *et al* .,1991) and Lactacin B (Barefoot and Klean Hammer, 1983).

The largest spectrum in this study was exhibit by *L. bulgaricus* Y34, *Lactococcus lactis* N22 and *S. thermophilus* N27 which inhibit all indicator microorganisms used in this study. This is in conformity with earlier reports by Tagg *et al*. (1976), Daesilel and Klean Hammer (1985) and Sanni *et al*. (1999) that some bacteriocins produced by gram-positive bacteria have broad spectrum activities. However it was generally observed that bacteriocins from the producer organisms had no inhibitory effects on the organisms producing it. The implication is that both the bacteriocin and the bacteriocin producing LAB could be used for biopreservation of foods without adverse effects.

## 5. CONCLUSIONS

In conclusion, it was observed that not all species of lactic acid bacteria are good bacteriocin producers as such those species of LAB identified as potential bacteriocin producers are recommended to food processing industries to be employed in biopreservation of foods to enhance extension of shelf life of food products and to reduce the risk of the use of chemical preservatives and additives, as they could pose health risk generally.

## 6. REFERENCES

[1] Adams, M.R. and Moss, M.O (1995). *Food Microbiology*. Royal Society of Chemistry, Gulford, pp. 252-284.

[2] Adams, M.R. and Nicolaides, L. (1997). *Review of the sensitivity of different Food borne pathogens to fermentation food control, U.K.*

[3] Axelson, L. (1998). Lactic acid bacteria: classification and physiology of lactic acid bacteria, *Microbiology and Functional Aspects.* Salminen, S. and Weight, A. (eds), Marcel Decker Inc., New York.

[4] Balasubramanyam, B.V. and Varadara, M.C. (1998). Cultural conditions for the production of bacteriocin by a native isolate *Lactobacillus delbruecki* sp. *Bulgaricus*. CRF 2018 in milk medium. *Journal of Applied Microbiology* 84:97-102.

[5] Bare foot, S.F. and Klaenhammer, T.K. (1983). Detections and activity of lactacin B on bacteriocin produced by *Lactobacillus acidophilus. Applied and Environmental Microbiology* 45: 1808-1815.

[6] Brinkten, B., Minekns, M., Vander Vossen, J.M., Leer, R.J. and Veld, J.H.J. (1994). Antimicrobial activity of lactobacilli. *Journal of Applied Bacteriology* 77:140-148.

[7] Casla, D, Requena, T. and Gomez, R. (1996). Antimicrobial activity of lactic acid bacteria isolated from goat's milk and artisanal cheeses: Characteristics of a bacteriocin produced by *Lactobacillus curvatus*. Journal of *Applied Bacteriology* 81: 35-41.

[8] Chatterjee, C., Paul, M., Xie, L. and Vander Donk, W.A.(2005). Biosynthesis and mode of action of lantibiotics. *Chemistry Review* 105:633-683.

[9] Cheese brough, M. (2003). *District Laboratory Practices in Tropical Countries*. Cambridge University Press, Edinburgh, pp. 382-407.

[10] Corsetti, A., Gobbetti, M. and Smacchi, E. (1996). Antimicrobial activity of Sour dough lactic acid bacteria: Isolation of bacteriocin-like inhibitory Substance from *Lactobacillus sanfrancisco* C57. *Food Microbiology* 13:447-456.

[11] Daba, H., Pandian, S., Gorsselinh, J.E., Huang, J. and Lacroix, C. (1991). Detection and activity of bacteriocin produced by *Leuconostoc mesenteroides*. *Applied Microbiology and Bacteriology* 39:166-173.

[12] Daeschel, M.A. (1993). Application and interactions of bacteriocins from lactic acid bacteria in foods and beverages. In: *Bacteriocin of Lactic Acid Bacteria:* Eds. Hoover, D.G and Steenson. L.R, Academic Press, London, pp. 63-91.

[13] Daeschel. M.A and Klaenhammer, T.R. (1985). Association of a 13.6 mega Dalton plasmid in *Pediococcus pentosaceus* with bacteriocin activity. *Applied and Environmental Microbiology* 50: 1538-1541.

[14] Delves-Broughton, J.(1990). Nisin and its use as food and preservative. *Food Technology* 44:100-117.
[15] De vugst, L. and Vandamme, E.J. (1994). Nisin, an antibiotic produced by *Lactococcus lactis subsp. lactis*: Properties, Biosynthesis, Fermentation and Application. In: *Bacteriocins of Lactic Acid Bacteria,* Blackie, London, pp.151-221.
[16] El-shafei, H.A., Abd-el-sabour, J.I., Ibrahim, M. and Mostafa, Y.A. (2000). Isolation, screening and characterization of bacteriocin producing lactic acid bacteria isolated from traditional fermented food. *Microbiology Review* 154 (4): 321-331.
[17] Fawole, M.O. and Oso, B.A. (1998). *Laboratory Manual of Microbiology*. Spectrum Book Limited, Ibadan, Nigeria, pp. 16-35.
[18] Graciela, M., Vignolo, M., de Kairuz, M., Aida, A.P., de Ruiz, H and Oliver, (1995). Influence of growth conditions on the production of bacteriocin by *L. Casei* CRL705. *Journal of Applied Bacteriology* 78: 5-10.
[19] Halo, H., Nilssen, O. and Nes, I.F. (1991). Lactococcin A, a new bacteiocin from *Lactococcus lactis sub sp. cremoris:* Isolation and characterization of the protein and its gene. *Journal of Bacteriology* 173: 3887.
[20] Hammes, W., Weiss, N and Holzeptel, W. (1999). *The Prokayotes.* Spinger-Velag, New York, pp 1-103.
[21] Lindgreen, S.W. and Dobrogeosz, W.L. (1990). Antagonistic activities of lactic acid bacteria in food and food fermentations. *Microbiology Review* 87:49-160.
[22] Menssens, W. and De vugst. (2002). Inhibitory substances produced by Lactobacilli isolated from Sourdought - A review *International Journal of Food Microbiology*72:31-43.
[23] Mohammed, S.S.D., IjahU.J.J., Baba,J., Bala,E., Damisa,D., Shanba,A.M., Muhammad,I.L., Muham Mad, R.G. and Kumar,N. ( 2012). Purification and Characterization of bacteriocins produced by *Pediococcus halophilus* W9 and *Lactococcus lactis* N24.*Developmental Journal of Science and Technology Research.*1 (1):85-96.
[24] Nettles, G.G. and Bare foot, S.F. (1993) Biochemical and genetic characteristics of bacteriocins of food associated lactic acid bacteria. *Journal of Food Production* 56: 338-356.
[25] Odunfa, S.A. (1985). African fermented foods. In. B.J.B Wood, ed. *Microbiology of Foods*. London: EL Sevier, pp 155-191.
[26] Ogunbanwo, S.T., Sanni, A.I. and Onilude, A.A. (2003). Influence of cultural conditions on the production of bacteriocin by *Lactobacillus brevis* OG1. *African Journal of Biotechnology* 7:179-184.
[27] Olatunji, E.A., Shiawoya, E.L. and Ijah, U.J.J. (2006) Bacterial assessment and keeping quality of local cheese (wara) in whey and salt solution. *Journal of Sustainable Tropical Agricultural Research,* 17:125-130.
[28] Olukoya, D.K. (1993). *Lactobacillus* in human health with reference to locally fermented foods. *Nigerian Med*ical *Journal* 24 (1):28-30.
[29] Oyeleke, S.B., Faruk, A.K., Oyewole, O.A. and Nabara H.Y.(2006) Occurrence of lactic acid bacteria in some locally fermented food products sold in Minna markets. *Nigerian Journal of Microbiology* 20(2):927-930.
[30] Oyeleke S. B. and Manga, B.S. (2008). *Essentials of Laboratory Practical in Microbiology* (first edition). Tobest Publishers, Minna, Nigeria, pp. 28 – 62.
[31] Remmelsberg, M. and Radler, F. (1990). Antimicrobial polypeptides of *Lactobacillus* species. *Journal of Applied Bacteriology* 69:177-184.
[32] Rodriguez, J.M., Cintas, L.M., Casaus, P., Suarez, A. and Hernandez, P. (1995). PCR detection of the lactocin S structural gene in bacteriocin- producing lactobacilli from meat. *Applied and Environment Microbiology* 61.2805.
[33] Sanni, A.I., Onilude, A.A., Ogunbanwo, S.T. and South, S.I. (1999). Antagonistic activity of bacteiocin produced by *Lactobacillus* species from Ogi, an indigenous fermented food. *Journal of Basic Microbiology* 39:189-195.
[34] Schillinger, V. and Lucke, F.K. (1989). Antimicrobial activity of *Lactobacillus sake* isolated from meat. *Applied and Environmental Microbiology* 55: 1901-1906.
[35] Tagg, J.R. Dajani, A.S. and Wannamaker, L.W. (1976). Bacteriocins of gram positive bacteria. *Bacteriology Review* 40: 722-756.
[36] Taylor, D.J., Green, N.P.O. and Stout, G.W. (1997). *Biological Science,* 3rd Ed. Cambridge University Press, Cambridge, pp. 401-402.
[37] Vingnolo, G.M., De Kairuz, M.N., De Ruiz Holgada, A.A.P. and Oliver, G. (1995). Influence of growth conditions on the production of Lactocin 705, a bacteriocin produced by *Lactobacillus casei* CRL 705. *Journal of Applied Bacteriology 78:5-9.*
[38] Vignolo, G. Fadda, S., De kairuz, M.N. De Ruiz Holgado, A.A.P. and Oliver, G. (1996). Control of *Listeria monocytogenes* in ground beef by Lactocin 705. a bacteriocin produced by *L. casei* CRL 705. *International Journal of Food Microbiology* 27: 397-402.